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Transcriptome analysis of the wheat–*Puccinia striiformis* f. sp. *tritici* interaction

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SUMMARY

Stripe rust [caused by *Puccinia striiformis* Westend. f. sp. *tritici* Eriks. (*Pst*)] is a destructive disease of wheat (*Triticum aestivum* L.) worldwide. Genetic resistance is the preferred method for control and the *Yr5* gene, originally identified in *Triticum spelta* var. *album*, represents a major resistance (*R*) gene that confers all-stage resistance to all currently known races of *Pst* in the United States. To identify transcripts associated with the *Yr5*-mediated incompatible interaction and the *yr5*-compatible interaction, the Wheat GeneChip was used to profile the changes occurring in wheat isolines that differed for the presence of the *Yr5* gene after inoculation with *Pst*. This time-course study (6, 12, 24 and 48 h post-inoculation) identified 115 transcripts that were induced during the *R*-gene-mediated incompatible interaction, and 73 induced during the compatible interaction. Fifty-four transcripts were induced in both interactions and were considered as basal defence transcripts, whilst 61 transcripts were specific to the incompatible interaction [hypersensitive response (HR)-specific transcripts] and 19 were specific to the compatible interaction (biotrophic interaction-specific transcripts). The temporal pattern of transcript accumulation showed a peak at 24 h after infection that may reflect haustorial penetration by *Pst* at ~16 h. An additional 12 constitutive transcript differences were attributed to the presence of *Yr5* after eliminating those considered as incomplete isogenicity. Annotation of the induced transcripts revealed that the presence of *Yr5* resulted in a rapid and amplified resistance response involving signalling pathways and defence-related transcripts known to occur during *R*-gene-mediated responses, including protein kinase signalling and the production of reactive oxygen species leading to a hypersensitive response. Basal defence also involved substantial induction of many defence-related transcripts but the lack of *R*-gene signalling resulted in weaker response.

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INTRODUCTION

Plant resistance (*R*) gene products are thought to recognize pathogen avirulence (*Avr*) gene products through either direct (receptor–ligand model) or indirect (guard hypothesis) association, triggering signal transduction cascades that lead to rapid defence mobilization (Dangl and Jones, 2001; Hammond-Kosack and Parker, 2003). Systems of active defence are known to include calcium and ion fluxes, increase of reactive oxygen species (ROS) during the oxidative burst (Lamb and Dixon, 1997) and hypersensitive cell death (hypersensitive response, HR) (Greenberg, 1997). Different forms of plant defence have been identified that include non-host, non-race-specific and race-specific resistance, as well as basal defence. According to the definitions of Hammond-Kosack and Parker (2003), race-specific resistance is R-protein-mediated, but resistance varies within a species and is only effective in genotypes that carry R proteins that recognize pathogen-specific elicitors. Conversely, basal defence refers to the response activated in a susceptible genotype of a host plant species, although this type of defence also occurs in resistant genotypes but does not condition resistance on its own.

Stripe rust [caused by *Puccinia striiformis* Westend. f. sp. *tritici* Eriks. (*Pst*)] is an important and destructive disease of wheat (*Triticum aestivum* L.) worldwide (Chen, 2005). Genetic resistance is the preferred method for controlling stripe rust and the *Yr5* gene, originally identified in *Triticum spelta* var. *album* (TSA) (Chen *et al.*, 1998; Lupton and Macer, 1962; McIntosh *et al.*, 1998), confers race-specific all-stage resistance to all currently known races of *Pst* in the United States (X. Chen, unpublished data) but is susceptible to some isolates from Australia (Wellings and McIntosh, 1990) and India (Nagarajan *et al.*, 1986). The infection process of *Pst* begins with non-fungistatic urediniospores that germinate on leaf surfaces by producing a germ tube that grows parallel to leaf veins. Germination on both susceptible and resistant plants occurs within 12 h post-inoculation (hpi) (Mares and Cousen, 1977). The fungus does not form appressoria and the germ tube directly penetrates the stoma to form a substomatal vesicle at 8–12 hpi (Moldenhauer *et al.*, 2006), followed by infection hyphae and haustorial formation at 16 hpi. It is not known whether resistance to *Pst* is pre- or

GeneChip datasets compared	Category
Yr5 Pst-inoculated—mock-inoculated	**1.** Incompatible interaction
	a. HR-specific transcripts
	b. Basal defence transcripts
yr5 Pst-inoculated—mock-inoculated	**2.** Compatible interaction
	a. Basal defence transcripts
	b. Biotrophic interaction-specific transcripts
Yr5 Pst-inoculated—*yr5 Pst*-inoculated	**3.** Constitutive *Yr5*-controlled transcripts
	a. *Pst*-specific *Yr5* transcripts
	b. *Pst*-specific *yr5* transcripts
	c. Incomplete isogenicity transcripts
Yr5 mock-inoculated—*yr5* mock-inoculated	**4.** Incomplete isogenicity transcripts

Table 1 Description of the four analysis categories and subcategories, and the GeneChip datasets used for each analysis. The first named dataset in each comparison was considered the experimental data, and the second named was the control (baseline) data.

post-haustorial, but wheat resistance to leaf rust has been shown to be post-haustorial (Kloppers and Pretorius, 1995).

To characterize the transcriptional changes involved during an incompatible race-specific resistance interaction and a compatible interaction with *Pst*, particularly those controlled by *Yr5*, we employed the recently developed Affymetrix GeneChip Wheat Genome Array. The array represents over 55 000 wheat transcripts from all chromosomes and ancestral genomes. We chose to profile the changes occurring in two wheat isolines that differed for the presence of *Yr5* over a time-course that spanned the predicted *Pst* urediniospore germination and infection process. Transcript changes attributed to differences in genotype were minimized by the use of isolines. The aim was not to characterize the *Yr5* gene itself, but to characterize the defence pathways and mechanisms controlled by the presence of *Yr5*. Subsequently, we identified 115 transcripts that were induced during the incompatible interaction with respect to mock-inoculated controls, and 73 for the compatible interaction. Twelve additional transcripts considered as constitutively *Yr5*-controlled were identified by directly comparing the GeneChip data of each isoline, which also allowed for an assessment of transcript changes caused by incomplete isogenicity. With the aim of facilitating map-based cloning of *Yr5*, the GeneChip data were also explored for the development of genetic markers that were linked to *Yr5*.

RESULTS

Fungal infection and disease development

Disease progression on both *Pst*-inoculated and mock-inoculated seedlings of the *Yr5* and *yr5* isolines was monitored until 20 days post-inoculation (dpi). For both isolines, leaves of all *Pst*-inoculated plants began to show chlorotic flecking at ~8 dpi, which was indicative of an HR. However, by ~14 dpi, heavy sporulation (formation of rust uredia) was observed on *Pst*-inoculated *yr5* plants, while plants of the *Yr5* isoline displayed only small necrotic flecks (data not shown). All mock-inoculated plants were free of any disease symptoms over the 20-day observation period.

Wheat GeneChip analysis strategy

Measurements and comparisons of transcript abundance in *Pst*-inoculated and mock-inoculated *Yr5* and *yr5* isolines at 6, 12, 24 and 48 hpi were performed using the Wheat GeneChip. Time-course hybridizations from each of four experimental conditions (*Yr5 Pst*-inoculated, *Yr5* mock-inoculated, *yr5 Pst*-inoculated and *yr5* mock-inoculated) were biologically replicated three times and all data were analysed using a combination of software modules. Correlation coefficients between replications ranged from 0.97 to 0.99. All biological replications were pooled and four separate comparisons (categories) were performed to identify transcripts involved in different aspects of the inoculation response (Table 1). For each category, probe sets were filtered for a fold change threshold of > 2.0 and a parametric two-way ANOVA ($P < 0.001$) with false discovery rate (FDR) multiple testing correction at 5% was applied to identify statistically significant differentially expressed probe sets. In order to focus on gene expression differences related to genotype or treatment, all probe sets significant only for the time effect were excluded from further analysis. Each probe set was assumed to represent a single transcript, and functional annotation of the corresponding unigenes was performed using HarvEST (Affymetrix Wheat1 Chip version 1.52). Gene ontology (GO) was based on the TIGR rice genome annotation.

Incompatible interaction responses

The first comparison category, which compared *Pst*-inoculated *Yr5* GeneChip data versus *Yr5* mock-inoculated data (Table 1 category 1), was assumed to provide information regarding overall race-specific resistance to *Pst*, including HR-specific transcripts and basal defence transcripts. A total of 116 transcripts were differentially expressed according to treatment and/or treatment $\times$ time interaction, of which 115 were induced by *Pst*-inoculation over the time course and only one was repressed (Table 2). The accumulation pattern of the 115 induced transcripts showed a peak at 24 hpi (data not shown). The distribution of biological

Table 2 List of the transcripts significantly induced and repressed by *Puccinia striiformis* f. sp. *tritici* (*Pst*) in reference to mock-inoculated controls in the incompatible (*Yr5*) interaction and the compatible (*yr5*) interaction after two-way ANOVA ($P < 0.001$, treatment effect only), FC > 2.0 cutoff, and FDR correction. Values represent mean $\log_2$ FC, GO refers to gene ontology, and putative function represents the best database hit from HarvEST

			Incompatible interaction				Compatible interaction			
Putative GO category	Putative function	Probe set	6 h	12 h	24 h	48 h	6 h	12 h	24 h	48 h
HR-specific transcripts										
Defence—cell wall	Proline-rich protein	Ta.16599.1.S1_at		1.44	2.39					
Defence—cell wall	Pathogen-induced protein WIR1A homologue	Ta.22732.1.S1_x_at			2.05	1.62				
Defence—cell wall	Pathogen-induced protein WIR1A homologue	Ta.3133.1.S1_x_at			2.86	3.67				
Defence—oxidative burst	Peroxidase	Ta.18497.1.S1_at		2.49	2.50					
Defence—oxidative burst	Peroxidase	Ta.24106.1.S1_x_at			1.87	1.36				
Defence—phenylpropanoid	Phenylalanine ammonia-lyase	Ta.7022.1.S1_at		1.35						
Defence—phenylpropanoid	Phenylalanine ammonia-lyase	TaAffx.131379.1.A1_at		2.44						
Defence—phenylpropanoid	Phenylalanine ammonia-lyase	TaAffx.92008.1.A1_s_at		2.35	1.79					
Defence—PR protein	Beta-1,3-glucanase	Ta.1174.1.S1_x_at			2.80	2.78				
Defence—PR protein	Beta-1,3-glucanase	Ta.21297.1.S1_at			2.56	1.95				
Defence—PR protein	Beta-1,3-glucanase	Ta.21354.1.A1_at				1.04				
Defence—PR protein	Beta-1,3-glucanase	Ta.21354.1.A1_x_at				1.27				
Defence—PR protein	PR protein 10	Ta.22619.1.S1_at			1.83	2.98				
Defence—PR protein	PR protein 10	Ta.22619.1.S1_x_at			2.15	3.46				
Defence—PR protein	Beta-1,3-glucanase	Ta.26048.1.S1_x_at			1.60	1.57				
Defence—PR protein	Thaumatin-like protein	Ta.27762.1.S1_x_at			1.66	2.71				
Defence—PR protein	Chitinase	Ta.30501.1.S1_at			2.61	3.88				
Defence—PR protein	Beta-1,3-glucanase	TaAffx.119315.2.S1_at			1.36					
Defence—PR protein	Beta-1,3-glucanase	TaAffx.119315.2.S1_x_at		1.03	1.41					
Energy—electron transport	Cytochrome P450	Ta.29826.1.S1_at			1.43					
Energy—electron transport	Cytochrome P450	Ta.8262.1.S1_at			2.00	1.46				
Energy—electron transport	Cytochrome P450	Ta.8447.1.S1_a_at	1.21	1.96	5.00	2.00				
Energy—electron transport	Cytochrome P450	Ta.8447.1.S1_x_at			3.13					
Energy—electron transport	Blue copper-binding protein	Ta.9336.1.S1_x_at			1.58					
Energy—electron transport	Cytochrome P450	TaAffx.109794.1.S1_s_at			2.86					
Growth—GA biosynthesis	Gibberellin oxidase	Ta.24934.3.S1_at		1.55	1.91	1.18				
Growth—GA biosynthesis	Ent-kaurene synthase	Ta.8418.1.S1_at			1.71	1.42				
Metabolism—carbohydrate	UDP-glucose dehydrogenase	Ta.2657.1.S1_x_at		1.08	2.17					
Metabolism—carbohydrate	SIS domain protein	Ta.4815.1.S1_at		1.13	1.42					
Metabolism—carbohydrate	Beta-fructofuranosidase	TaAffx.82312.1.S1_s_at		1.10	1.16					
Signal transduction	LRR-containing extracellular glycoprotein	Ta.27314.1.S1_at			1.96	1.67				
Signal transduction	Secretory protein kinase	TaAffx.52945.1.S1_at		1.45	2.30					
Transport—carbohydrate	Glucose transporter	Ta.12517.1.S1_at			1.21					
Transport—carbohydrate	Sugar transporter	Ta.27329.1.S1_at			1.34					
Unclear	NA	Ta.13991.1.S1_x_at		1.15	2.50	1.51				
Unclear	NA	Ta.14231.1.S1_x_at			1.25					
Unclear	NA	TaAffx.107538.1.S1_x_at			1.48					
Unclear	NA	TaAffx.110081.1.S1_at			1.47	1.41				
Unclear	NA	TaAffx.110081.1.S1_x_at			1.46	1.57				
Unclear	NA	TaAffx.110250.1.S1_x_at		1.20	1.78					
Unclear	NA	TaAffx.7302.1.S1_at			1.79	1.07				
Unknown	NA	Ta.11087.2.S1_at				1.07				
Unknown	NA	Ta.11087.2.S1_x_at				1.42				
Unknown	NA	Ta.14618.1.A1_at			−1.60					
Unknown	NA	Ta.21236.1.S1_a_at		1.42	3.04					
Unknown	NA	Ta.21236.3.S1_x_at		1.83	3.10					
Unknown	NA	Ta.21314.1.S1_at		2.46	3.98	2.74				

Table 2 *continued.*

			Incompatible interaction				Compatible interaction			
Putative GO category	Putative function	Probe set	6 h	12 h	24 h	48 h	6 h	12 h	24 h	48 h
Unknown	NA	Ta.21314.1.S1_x_at		2.45	3.83	2.76				
Unknown	NA	Ta.22223.1.S1_at		1.18	1.48					
Unknown	NA	Ta.22957.1.S1_at		2.17	3.84	1.64				
Unknown	NA	Ta.3247.1.S1_at			1.04					
Unknown	NA	Ta.8254.1.A1_at			2.44	1.18				
Unknown	NA	Ta.8582.1.S1_at	1.05	1.88	3.35					
Unknown	NA	Ta.8582.2.S1_a_at			2.18					
Unknown	NA	Ta.8582.2.S1_x_at		1.26	2.67					
Unknown	NA	TaAffx.108939.1.S1_at			1.81					
Unknown	NA	TaAffx.109709.1.S1_at			1.37					
Unknown	NA	TaAffx.109765.1.S1_at		1.07	2.07					
Unknown	NA	TaAffx.27177.1.S1_at				1.41				
Unknown	NA	TaAffx.52926.1.S1_at		1.72	2.38					
Unknown	NA	TaAffx.55533.1.S1_at			1.22					
Unknown	NA	TaAffx.82674.1.S1_at		1.08						
Biotrophic interaction-specific transcripts										
Defence—lignin	Dirigent-like protein	Ta.11506.1.S1_a_at								–1.56
Defence—phenylpropanoid	Phenylalanine ammonia-lyase	Ta.7022.1.S1_x_at							1.34	
Energy—electron transport	Blue copper-binding protein	Ta.5654.1.S1_at							1.71	
Energy—electron transport	Blue copper-binding protein	Ta.8483.1.S1_at							1.13	
Metabolism—carbohydrate	Nudix hydrolase	Ta.18888.1.S1_at							1.18	
Metabolism—carbohydrate	Polygalacturonase	Ta.8782.1.S1_at							2.64	
Metabolism—galactose	Aldose 1-epimerase	Ta.10680.1.S1_at							1.41	
Metabolism—glycerol	Glycerophosphoryl diester phosphodiesterase	TaAffx.113645.2.S1_at							1.19	
Metabolism—sterol	Cytochrome P450 51	TaAffx.107979.1.S1_at							3.03	
Metabolism—sterol	Cytochrome P450 51	TaAffx.28047.1.S1_at							4.54	
Metabolism—sterol	Cytochrome P450 51	TaAffx.28047.1.S1_s_at							1.27	
Signal transduction	Protein kinase	Ta.25487.1.S1_at							1.22	
Transport	HIPL1 protein	Ta.5666.1.S1_at					1.26	1.15		
Unclear	NA	Ta.22482.1.S1_s_at							1.00	
Unclear	NA	Ta.25140.1.S1_at					1.22	1.24	1.19	
Unclear	NA	Ta.806.2.A1_a_at								–1.17
Unclear	NA	TaAffx.53352.1.S1_x_at							1.78	1.08
Unclear	NA	TaAffx.78553.1.S1_at							1.06	
Unknown	NA	Ta.16165.1.S1_at							2.24	1.17
Unknown	NA	Ta.27882.1.S1_x_at							1.17	
Unknown	NA	TaAffx.7236.1.S1_at						1.05	2.63	
Basal defence transcripts										
Defence	Pathogen-induced secretory protein	Ta.231.1.S1_at			1.29				2.03	
Defence	Pathogen-induced secretory protein	Ta.231.1.S1_x_at			1.71				2.58	
Defence—alkaloid	Reticuline oxidase	Ta.27350.1.S1_at		1.59	1.46			1.32	2.00	
Defence—anthocyanin	UDP-glycosyltransferase	Ta.23340.1.S1_at		2.55	5.17	3.85		1.41	5.28	2.79
Defence—anthocyanin	UDP-glycosyltransferase	Ta.30731.1.S1_at		1.69	4.89	3.47			5.33	2.26
Defence—lignin	Dirigent-like protein	Ta.22687.1.A1_at		1.13	2.24	1.40			2.47	
Defence—lignin	Cinnamyl-alcohol dehydrogenase	Ta.28562.1.A1_at		1.09	2.38				2.89	
Defence—lignin	Caffeic acid 3-O-methyltransferase	Ta.336.1.S1_x_at			2.05				2.10	
Defence—lignin	Caffeic acid 3-O-methyltransferase	Ta.336.2.S1_at			1.75				2.05	

Table 2 *continued.*

			Incompatible interaction				Compatible interaction			
Putative GO category	Putative function	Probe set	6 h	12 h	24 h	48 h	6 h	12 h	24 h	48 h
Defence—lignin	Dirigent-like protein	TaAffx.28302.2.S1_at		1.44	3.01	1.98			3.31	
Defence—oxidative burst	Peroxidase	Ta.22564.1.S1_at			4.01	1.36			4.49	1.08
Defence—oxidative burst	Peroxidase	Ta.22564.2.S1_a_at			3.82	1.20			4.22	1.12
Defence—oxidative burst	Peroxidase	Ta.22564.2.S1_x_at			3.34	1.09			3.86	
Defence—oxidative burst	Peroxidase	Ta.5385.1.S1_at	1.21		3.34	3.81	1.13	1.38	4.06	3.41
Defence—oxidative burst	Peroxidase	Ta.82.1.S1_at		2.12	4.61	2.38			4.47	1.96
Defence—phenylpropanoid	Phenylalanine ammonia-lyase	Ta.1465.1.S1_at			1.80				2.51	
Defence—phenylpropanoid	Phenylalanine ammonia-lyase	Ta.7022.1.S1_s_at	1.07	2.91	1.90			1.55	2.19	
Defence—phenylpropanoid	Phenylalanine ammonia-lyase	Ta.9220.1.S1_a_at		3.01	2.61		1.27	1.72	3.11	
Defence—PR protein	PR protein 1	Ta.13013.2.S1_x_at			1.27			1.10	1.24	
Defence—PR protein	Beta-1,3-glucanase	Ta.20750.1.S1_at		1.87	2.34	1.50		1.46	2.49	
Defence—PR protein	Beta-1,3-glucanase	Ta.22562.1.S1_at	1.61	2.05	3.61	3.03	1.86	1.79	3.32	2.14
Defence—PR protein	Beta-1,3-glucanase	TaAffx.131249.1.S1_at		2.14	2.14	1.31		1.75	2.63	1.01
Defence—PR protein	Beta-1,3-glucanase	TaAffx.131249.1.S1_s_at	1.22	2.14	2.62	1.56	1.50	1.81	2.96	1.12
Energy—electron transport	Cytochrome P450	TaAffx.107507.1.S1_at			3.64				4.19	
Energy—electron transport	Blue copper-binding protein	TaAffx.55612.1.S1_at		1.09	1.61				1.58	
Metabolism—sterol	Cytochrome P450 51	Ta.8346.1.A1_at		1.03	1.32				2.23	
Signal transduction	Protein kinase	Ta.27258.1.S1_at			1.80	1.23			1.85	
Signal transduction	Protein kinase	TaAffx.23165.2.S1_at			1.56				1.70	
Signal transduction	Protein kinase	TaAffx.23205.1.S1_at			1.41				1.84	
Signal transduction	Secretory protein kinase	TaAffx.52945.3.S1_at			1.18				1.55	
Signal transduction	Secretory protein kinase	TaAffx.52945.3.S1_x_at		1.28	2.06				2.15	
Transport	Phosphate transporter	Ta.10084.1.S1_at			1.70				1.67	
Transport	Sugar transporter	Ta.1760.1.S1_at		1.13	1.83				1.93	
Transport	Sugar transporter	Ta.1760.1.S1_x_at		1.07	1.86				1.93	
Transport	Amino acid permease	Ta.22300.1.S1_at		1.46	2.35			1.27	3.09	
Transport	Agmatine coumaroyltransferase	Ta.8228.1.S1_at			1.55				3.03	
Transport	Peptide transporter	Ta.8459.1.A1_at		2.17	1.88			1.73	2.52	
Unclear	NA	TaAffx.109424.1.S1_at		2.47	3.51	1.02		1.59	3.21	
Unknown	NA	Ta.10589.1.S1_at		1.35	2.49			1.02	3.09	
Unknown	NA	Ta.12795.1.S1_at	1.15	1.56	2.23		1.23		1.75	
Unknown	NA	Ta.1454.3.S1_at			1.37	1.07			1.65	
Unknown	NA	Ta.16472.1.S1_s_at		1.72	2.20			1.46	2.62	
Unknown	NA	Ta.16472.1.S1_x_at			1.04				1.88	
Unknown	NA	Ta.21236.2.S1_x_at		1.81	2.90		1.01	1.58	3.05	
Unknown	NA	Ta.23165.2.S1_x_at			1.23	2.10	1.24		1.58	1.23
Unknown	NA	Ta.23165.3.S1_x_at			1.25	1.89	1.21		1.47	1.11
Unknown	NA	Ta.23340.2.S1_at		2.33	5.00	3.06		1.40	4.97	2.46
Unknown	NA	Ta.27882.1.S1_s_at			1.49	1.14			1.54	
Unknown	NA	Ta.30829.1.S1_at			1.22				1.85	
Unknown	NA	TaAffx.104648.1.S1_at	1.34	3.23	4.25	1.80	1.23	2.82	3.82	
Unknown	NA	TaAffx.26815.1.S1_at		1.55	1.81				2.04	
Unknown	NA	TaAffx.32249.1.S1_at	3.92	3.64	4.42	2.94	5.20	5.52	5.27	4.16
Unknown	NA	TaAffx.53805.1.S1_at			1.19				1.92	
Unknown	NA	TaAffx.59551.1.S1_at	2.56	2.73	2.78	1.43	3.36	3.88	3.26	1.89

function for the *Pst*-induced transcripts included 42 (37%) transcripts putatively involved in pathogen defence-related pathways, including phenylpropanoid biosynthesis (anthocyanins and lignin), oxidative stress and pathogenesis-related (PR) proteins. Seven (6%) were involved in signal transduction, including protein kinases that may be involved in defence signalling. A further eight (7%) transcripts were involved in protein/carbohydrate transport, eight (7%) in electron transport, six (5%) in growth and metabolism, and 44 (38%) were unknown (no matching database entry) or unclear (matched to a hypothetical protein only) transcripts. The single repressed transcript was of unknown function.

Compatible interaction responses

The comparison of *yr5 Pst*-inoculated with mock-inoculated data (Table 1 category 2) revealed gene expression changes induced during a compatible interaction that included basal defence transcripts and transcripts specific to the establishment of a biotrophic interaction with *Pst*. Seventy-three of the 75 differentially expressed transcripts significant for treatment and/or treatment × time interaction were induced by *Pst* inoculation, whilst just two were repressed (Table 2). Of the 73 induced transcripts, the peak of differential accumulation also occurred at 24 hpi (data not shown). Defence-related transcripts accounted for 24 (33%) of the induced transcripts, while six (8%) were involved in signal transduction, eight (11%) in metabolism, seven (10%) in protein/carbohydrate transport, four (5%) in electron transport and 24 (33%) were unknown/unclear. Of the two repressed transcripts, one was unclear and the other was defence-related with specific involvement in lignin biosynthesis.

Comparison of the transcripts induced by *Pst* during the incompatible (*Yr5*) and compatible (*yr5*) interactions with mock-inoculated controls revealed that a large proportion were induced in both interactions and were considered as basal defence transcripts (Fig. 1). Sixty-one transcripts were specifically induced during the incompatible interaction, which were termed as HR-specific (Fig. 1; Table 2). The HR-specific transcripts included many that were defence-related and unclear/unknown, as well as some metabolic, energy, growth, and transport-related transcripts. The 19 transcripts only induced in the compatible interaction were probably involved in the establishment of a biotrophic interaction with *Pst*. Most of these were metabolic and unclear/unknown transcripts, and only two were putatively involved in defence or signal transduction.

Constitutive *Yr5*-controlled transcripts and incomplete isogenicity

Comparing the *Pst*-inoculated GeneChip data from *Yr5* to *yr5* (Table 1 category 3) provided information regarding transcript expression that may reflect constitutive *Yr5*-controlled differences in the genomes of each isoline, or incomplete isogenicity. Using *yr5* data as a reference, 67 transcripts significant for genotype and/or genotype × time interaction effect were found to be constitutively expressed at a significantly higher level in *Yr5* samples, whilst 62 were expressed constitutively more in *yr5* samples. Of the 67 significant for *Yr5*, most were of unclear/unknown function (61%) and 18% were involved in defence, signal transduction or transcriptional regulation. Of the 63 significant *yr5* transcripts, 55% were unclear/unknown and 27% were involved in defence, signal transduction or transcriptional regulation.

To investigate and eliminate constitutive gene expression differences potentially caused by incomplete isogenicity, transcripts significantly different between isolines under mock-inoculation were identified (Table 1 category 4). These transcripts were assumed to represent broader differences in the genetic backgrounds of the isolines as they were found to be differentially expressed in the absence of *Pst*-inoculation. We identified 63 constitutive *Yr5* transcripts under mock-inoculation (see supplementary Table S1), most of which were of unclear/unknown function (63%). For *yr5*, 53 constitutive transcripts were identified (supplementary Table S1), with 60% representing unclear/unknown transcripts. These 'incomplete isogenicity' changes (Table 1 category 4) overlapped substantially with the constitutive *Yr5*-controlled transcripts detected under *Pst*-inoculation (Table 1 category 3). For *Yr5*, 55 transcripts were shared between both of the categories, leaving 12 *Pst*-specific *Yr5* transcripts that may be controlled specifically by *Yr5* rather than incomplete isogenicity (Table 3). Forty-seven of the *yr5 Pst*-inoculation transcripts were also common between the two categories, which left 16 *Pst*-specific *yr5* transcripts (Table 3) that may be controlled by the absence of *Yr5*. It is also possible that the *Yr5* locus controls some incomplete isogenicity transcripts, although this is unlikely. Finally, none of the significant transcripts for both of these categories was significant in the incompatible or compatible interactions, and thus represented true constitutive differences.

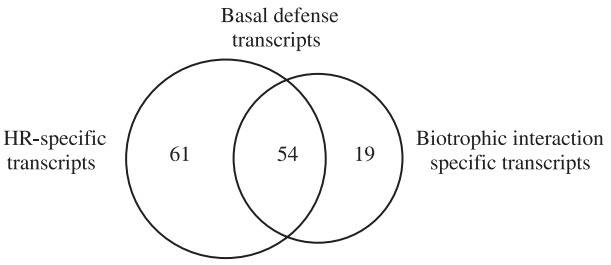

Fig. 1 The number of significant *Puccinia striiformis* f. sp. *tritici*-induced transcripts, in reference to mock-inoculated controls, that were shared and independent to each isoline. Significant transcripts were identified by two-way ANOVA ($P < 0.001$, genotype effect only), FC > 2.0 cutoff, and FDR correction.

Quantitative RT-PCR

To confirm the reliability of results from the GeneChip expression data, comparisons between GeneChip and quantitative RT-PCR (qRT-PCR) $\log_2$ expression ratios were made for 23 differentially expressed probe sets. The probe sets were selected to represent each of the comparison categories of Table 1, and also to obtain a cross-section of putative GO functional categories. All probe sets were validated with a PCR efficiency of 85–100%, and thus the results were compared without adjustment. Comparisons were made at 24 and 48 hpi and the expression ratios of 86% of the significant transcripts ($P < 0.001$ and fold change > 2.0) from the GeneChip analysis were confirmed (Table 4). The expression

Table 3 List of the *Puccinia striiformis* f. sp. *tritici* (*Pst*)-specific *Yr5* and *yr5* transcripts identified as part of the constitutive *Yr5*-controlled transcripts category, following two-way ANOVA ($P < 0.001$, genotype effect only), FC > 2.0 cutoff, and FDR correction. Values represent mean $\log_2$ FC of *Yr5* in reference to *yr5* such that positive values indicate probe sets expressed at a higher level in *Yr5* and negative values are those at a higher level in *yr5* samples. GO refers to gene ontology, and putative function represents the best database hit from HarvEST.

			Mean $\log_2$ FC			
Putative GO category	Putative function	Probe set	6 h	12 h	24 h	48 h
Pst-specific Yr5 transcripts						
DNA catabolism	S1/P1 Nuclease	Ta.5497.1.A1_at			1.19	
DNA catabolism	S1/P1 Nuclease	TaAffx.128682.1.S1_at			1.45	
Growth—cell replication	Chromosome condensation factor	Ta.2933.2.S1_x_at			1.18	
Signal transduction	Calmodulin-binding protein	Ta.23807.10.S1_at		1.03		1.05
Signal transduction	Calcium-binding EF hand protein	Ta.4155.1.S1_s_at		1.18		
Transcription	Myb transcription factor	Ta.25545.1.S1_at			1.42	
Unclear	NA	Ta.30565.1.A1_x_at			1.07	
Unclear	NA	Ta.9906.3.A1_a_at	1.03			
Unclear	NA	TaAffx.24957.1.S1_at				1.13
Unknown	NA	TaAffx.111269.1.S1_at				1.25
Unknown	NA	TaAffx.84234.1.S1_x_at			1.27	
Unknown	NA	TaAffx.9566.1.S1_at		1.60	1.38	
Pst-specific yr5 transcripts						
Defence	Subtilisin-chymotrypsin inhibitor 2	Ta.14230.1.S1_at		−1.44	−2.09	
Defence—*R* protein	NB-ARC domain containing protein	Ta.25981.1.A1_at			−1.32	
Metabolism	Inorganic pyrophosphatase	Ta.9458.2.S1_at	−1.07	−3.26	−3.57	−1.40
Metabolism—protein	F-box family protein	TaAffx.59384.1.S1_at		−1.01		
Signal transduction	GTP1/OBG family protein	Ta.16040.1.A1_x_at		−1.04	−1.60	
Signal transduction	Protein kinase	Ta.26120.1.S1_at			−1.09	
Signal transduction	Protein kinase	Ta.26120.1.S1_x_at			−1.12	
Transcription	MYND finger family protein	TaAffx.129136.1.S1_at	−1.15	−1.41	−1.29	
Transport	Agmatine coumaroyltransferase	Ta.8228.1.S1_at			−2.18	
Unclear	NA	TaAffx.128510.8.A1_at	−1.17	−1.16		
Unknown	NA	Ta.21783.1.S1_at	−1.04			
Unknown	NA	Ta.9866.1.S1_at	−1.06			
Unknown	NA	TaAffx.27427.1.S1_at	−1.02		−1.21	
Unknown	NA	TaAffx.31781.1.S1_at				−1.37
Unknown	NA	TaAffx.94040.1.A1_at			−1.09	

ratios observed for qRT-PCR tended to show greater differences in transcript levels than from the GeneChip analyses, which has been reported in other studies (Coram and Pang, 2006, 2007; Dowd *et al.*, 2004). Of all 56 comparisons between GeneChip and qRT-PCR, the direction of regulation (up or down) was conserved, which confirms the reliability of the GeneChip data.

DISCUSSION

The Wheat GeneChip was used to profile the transcript accumulation patterns in two wheat isolines that differed for the presence of the *Yr5* resistance gene. The comparison of GeneChip data within each isoline (*Pst*-inoculated vs. mock-inoculated) allowed us to examine the gene expression patterns involved in an incompatible (*Yr5*) and compatible (*yr5*) interaction, respectively. Further comparison of GeneChip data between isolines allowed us to identify constitutive *Yr5*-controlled transcripts (*Pst*-specific *Yr5* transcripts) and transcript differences likely to be due to incomplete isogenicity. Significant differentially expressed transcripts were identified using strict selection criteria that included a > 2.0-fold change threshold, two-way ANOVA ($P < 0.001$) and FDR multiple testing correction at the 5% level. Correlation coefficients between biological replications were > 0.97 and comparisons between GeneChip and qRT-PCR results revealed common expression kinetics for all results, indicating the strong reliability of the GeneChip data. Because the exact timing of the *Yr5* gene-for-gene interaction after *Pst* infection is not known, we used a time-course that spanned predicted *Pst* germination and penetration. The correlation between predicted haustorial penetration at ~16 hpi (Moldenhauer *et al.*, 2006), the transcriptional peak at 24 hpi and the identification of 61 HR-specific transcripts suggests that the gene-for-gene interaction may have taken

Table 4 Expression ratios of selected probe sets assessed by GeneChip (*Array*) and qRT-PCR (*qPCR*). Array values indicate mean $\log_2$ fold change with standard error, relative to the control (baseline) data of the particular category. qRT-PCR values indicate $\log_2$ ratios of $2^{\wedge}(\Delta C_t\text{control}/\Delta C_t\text{treatment})$. Array values followed by an asterisk are those that were significant after two-way ANOVA ($P < 0.001$, treatment effect only), FC > 2.0 cutoff, and FDR correction.

			Mean $\log_2$ fold change			
			24 hpi		48 hpi	
Putative function	Probe set	Category†	Array	qPCR	Array	qPCR
Beta-1,3-glucanase	Ta.1174.1.S1_x_at	1	2.80 ± 0.08*	2.15 ± 0.12	2.78 ± 0.47*	1.88 ± 0.06
Peroxidase	Ta.18497.1.S1_at	1	2.50 ± 0.35*	1.61 ± 0.35	0.11 ± 0.15	2.52 ± 0.02
Unknown	Ta.8582.1.S1_at	1	3.35 ± 0.53*	4.48 ± 0.03	0.59 ± 0.11	2.74 ± 0.03
LRR-containing extracellular glycoprotein	Ta.27314.1.S1_at	1	1.96 ± 0.27*	3.63 ± 0.05	1.67 ± 0.02*	2.81 ± 0.14
Cytochrome P450	Ta.8447.1.S1_a_at	1	5.00 ± 0.06*	6.26 ± 0.01	2.00 ± 0.18*	3.47 ± 0.07
Secretory protein kinase	TaAffx.52945.1.S1_at	1	2.30 ± 0.10*	2.00 ± 0.09	0.68 ± 0.08	0.77 ± 0.61
Phenylalanine ammonia-lyase	TaAffx.92008.1.A1_s_at	1	1.79 ± 0.06*	0.59 ± 0.36	–0.08 ± 0.01	–0.34 ± 1.37
Membrane-like protein	Ta.25140.1.S1_at	2	1.19 ± 0.21*	1.40 ± 0.14	0.27 ± 0.07	1.67 ± 0.19
Protein kinase	Ta.25487.1.S1_at	2	1.22 ± 0.02*	1.41 ± 0.25	0.05 ± 0.02	1.70 ± 0.13
HIPL1 protein	Ta.5666.1.S1_at	2	0.58 ± 0.27	0.70 ± 0.16	0.38 ± 0.04	0.52 ± 0.08
Cytochrome P450 51	TaAffx.28047.1.S1_at	2	4.54 ± 0.40*	6.21 ± 0.01	0.12 ± 0.24	1.21 ± 0.18
Calmodulin-binding protein	Ta.23807.10.S1_at	3	0.65 ± 0.06	1.31 ± 0.12	1.05 ± 0.05*	0.88 ± 0.22
Myb transcription factor	Ta.25545.1.S1_at	3	1.42 ± 0.19*	0.38 ± 0.15	0.64 ± 0.28	0.58 ± 0.29
Chromosome condensation factor	Ta.2933.2.S1_x_at	3	1.18 ± 0.04*	2.16 ± 0.13	0.47 ± 0.17	0.37 ± 0.29
Unclear	Ta.30565.1.A1_x_at	3	1.07 ± 0.07*	3.17 ± 0.02	0.15 ± 0.01	2.63 ± 0.06
Calcium-binding protein	Ta.4155.1.S1_s_at	3	0.67 ± 0.05	0.04 ± 0.50	0.30 ± 0.06	0.85 ± 0.28
Unclear	Ta.9906.3.A1_a_at	3	0.83 ± 0.06	2.63 ± 0.02	0.69 ± 0.02	1.83 ± 0.08
S1/P1 Nuclease	TaAffx.128682.1.S1_at	3	1.45 ± 0.18*	0.35 ± 0.15	0.57 ± 0.07	0.53 ± 0.18
Unknown	TaAffx.84234.1.S1_x_a	3	1.27 ± 0.01*	1.21 ± 0.09	0.44 ± 0.01	1.55 ± 0.21
Nitrate-induced NOI protein	Ta.20061.1.S1_at	3	0.65 ± 0.09	0.67 ± 0.46	1.36 ± 0.02*	1.31 ± 0.20
		4	0.82 ± 0.03	1.43 ± 0.10	1.08 ± 0.01*	1.66 ± 0.20
Pectinacetylesterase	Ta.23400.1.S1_at	3	1.50 ± 0.12*	1.91 ± 0.21	1.23 ± 0.27*	1.46 ± 0.72
		4	1.04 ± 0.09*	1.79 ± 0.51	1.11 ± 0.06*	1.05 ± 0.44
AP2 transcription factor	Ta.27144.1.S1_a_at	3	0.63 ± 0.13	1.87 ± 0.06	0.73 ± 0.23	1.09 ± 0.09
		4	0.99 ± 0.03	0.89 ± 0.38	0.29 ± 0.09	0.21 ± 0.08
Protein kinase	TaAffx.27775.1.S1_at	3	3.14 ± 0.35*	13.34 ± 0.01	4.54 ± 0.30*	8.75 ± 0.01
		4	2.95 ± 0.32*	7.20 ± 0.01	4.08 ± 0.27*	10.85 ± 0.01

†Datasets used for comparison (treatment vs. baseline).

1 = *Yr5 Pst*-inoculated *vs.* mock-inoculated.

2 = *yr5 Pst*-inoculated *vs.* mock-inoculated.

3 = *Yr5 Pst*-inoculated *vs. yr5 Pst*-inoculated.

4 = *Yr5* mock-inoculated *vs. yr5* mock-inoculated.

place within the time frame of this study. However, it is possible that the time-course did not capture the full complement of *Yr5*-controlled responses that may occur at later time-points.

Of the 115 transcripts induced during the incompatible interaction, 44% were also induced during the compatible interaction, and thus were classified as basal defence-related. Considering this overlap, effective resistance to *Pst* is likely to involve a combination of basal and *R* gene-mediated defences. In fact, it has been postulated that the difference between basal and *R* gene-mediated defence is simply the level of expression of the same pathway (Ellis *et al.*, 2007). A study of the soybean response to Asian soybean rust controlled by the *Rpp2* gene also found that incompatible and compatible interaction responses were similar in the early stages after infection (Van de Mortel *et al.*, 2007). Consistent with the results from their study, the temporal pattern of transcript accumulation during both interactions indicated a transcriptional peak around the time of fungal penetration. In our study the peak occurred at 24 hpi, which reflects haustorial penetration by *Pst* at ~16 hpi (Moldenhauer *et al.*, 2006). After this peak, Van de Mortel *et al.* (2007) observed a sharp decline in differential expression around 72–96 hpi, followed by another increase from 72 to 168 hpi. We observed decreasing differential expression at 48 hpi, but the time-course of this study did not allow us to detect a potential biphasic response. Another study of the barley response to powdery mildew controlled by the *Mla6*, *Mla13* and *Mla1* single resistance genes also found evidence for

a shared response between compatible and incompatible interactions up to the point of pathogen penetration (Caldo *et al.*, 2004). As also reported in these studies, we found numerous transcripts induced during the incompatible and compatible interactions that correspond to active systems of defence. Annotation of the basal defence transcripts showed evidence for defence signalling via protein kinases, expression of the oxidative burst, as well as synthesis of PR proteins and phenylpropanoids (anthocyanins and lignin). However, inclusion of the HR-specific transcripts in the incompatible interaction conferred a greater diversity of transcripts related to these defences than basal defence, which has been observed in other studies of this type (Caldo *et al.*, 2004; Hulbert *et al.*, 2007; Van de Mortel *et al.*, 2007). Considering that the majority of HR-specific transcripts were associated with defence, many unclear/unknown transcripts may also be defence-related.

To discover additional important transcripts based on the presence of *Yr5*, we identified constitutive *Yr5*-controlled transcripts by comparing *Pst*-inoculation results from the *Yr5* isoline with the *yr5* isoline (Table 1 category 3). However, these transcripts may also represent a broader differing genetic background between isolines, so we also identified transcripts that were significant under mock-inoculation that were probably caused by incomplete isogenicity (Table 1 category 4). Subsequently, after subtracting potential 'incomplete isogenicity' transcripts, we identified 12 constitutive *Pst*-specific *Yr5* transcripts that were more likely to be specific to *Yr5*-mediated resistance. These transcripts may represent genes controlled directly by *Yr5* and, interestingly, a high proportion of these were homologous to regions of rice chromosome 4L, which is syntenous with wheat chromosome 2BL (*Yr5* locus). Subsequently, we mapped one of these transcripts within 9.6 cM of the *Yr5* locus (data not shown). Three constitutive *Pst*-specific *Yr5* transcripts were also involved in signal transduction and/or transcriptional regulation, and may be responsible for activating the more diverse defence response observed in the incompatible interaction. It is also important to recognize that some of the significant transcripts in the 'incomplete isogenicity' category may also be important, and could actually represent the *Yr5* gene. However, this would be unlikely given that *Yr5* was originally sourced from TSA, which was not included in the *Triticum aestivum* Unigene build #38 (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nigene) used to construct the Affymetrix Wheat GeneChip. Furthermore, none of the significant *Yr5* transcripts of this category was homologous to known *R* gene motifs, and we therefore concluded that they were unlikely to represent the *Yr5* gene. However, of particular interest may be the two nitric oxide-induced (NOI) protein transcripts that were constitutively expressed at higher levels in *Yr5*, one (TaAffx.55202.1.S1_at) under only mock-inoculation, and the other (Ta.20061.1.S1_at) under both *Pst*- and mock-inoculation. Although this result represents a potential incomplete isogenicity difference between isolines, it may allude to the ability of *Yr5* to accumulate elevated cellular NO levels in response to both *Pst*- and mock-inoculation. Although candidate nitric oxide synthase probe sets were present on the GeneChip, hybridization to these probe sets was unsuccessful so we could not assess direct NO production.

The rapid accumulation of NO, together with ROS, is known to occur after pathogen challenge and lead to an oxidative burst and HR (Delledonne, 2005). The induction of two HR-specific peroxidase transcripts in addition to the five basal defence peroxidase transcripts provided evidence for an oxidative burst in this study that may contribute to the phenotypic HR observed in the incompatible interaction. Peroxidases have previously been reported as becoming induced during the resistance response of wheat to the fungal pathogen *Fusarium graminearum* (Mohammadi and Kazemi, 2002; Pritsch *et al.*, 2000). It has been proposed that peroxidases trigger subsequent NADPH oxidase-mediated ROS production and defence gene signalling during *R*-gene-mediated resistance and basal defence (Bindschedler *et al.*, 2006), which supports our observation of both HR-specific and basal defence peroxidase induction. Following *R*-gene-mediated pathogen perception, protein kinase (PK) activation is thought to be among the earliest events (Garcia-Brugger *et al.*, 2006). If *Yr5* is similar to other conserved plant *R* genes, such as the wheat leaf rust *R* genes *Lr10* (Feuillet *et al.*, 2003) and *Lr21* (Huang *et al.*, 2003), it is likely to perceive and then transduce the perception signal by a PK cascade and/or activation of transcription factors (TFs). This study does provide evidence for such an event, as several HR-specific and basal defence transcripts represented putative protein kinases, and two *Pst*-specific *Yr5* transcripts were homologous to an myb-like TF and a Ca^{2+}-binding signal transduction protein, respectively. Increased signalling ability may confer a more rapid defence response during the incompatible interaction, as would be expected. We also found one calmodulin (CaM) transcript as a *Pst*-specific *Yr5* transcript, which are known to act as Ca^{2+} receptors and transmit Ca^{2+} signals (Bouche *et al.*, 2005). In this study, we also found transcripts encoding several PR proteins, including beta-1,3-glucanse, chitinase, PR-10 and a thaumatin-like protein, to be HR-specific transcripts. PR proteins such as beta-1,3-glucanase and chitinase are also implicated in wheat resistance to *F. graminearum* (Boddu *et al.*, 2006), and thus may be important for resistance to multiple pathogens. Furthermore, the HR-specific induction of a proline-rich protein, which are structural proteins of the primary cell wall involved in restricting pathogen penetration (Otte and Barz, 2000), may be controlled by ROS. In chickpea, the H_2O_2 from an elicitor-induced oxidative burst has been shown to control directly the insolubilization of a proline-rich protein in cell walls and be induced in response to a fungal pathogen (Coram and Pang, 2006; Otte and Barz, 1996), and thus the proline-rich protein in this study may be induced by the oxidative burst and be effective in limiting *Pst* penetration during an incompatible interaction.

Numerous cytochrome P450 (CYP) transcripts were found in the HR-specific transcripts category. Although CYPs are involved in oxidative metabolism, some biosynthetic CYPs are known to participate in the synthesis of defence compounds including lignin, phytoalexins and anthocyanins (Sculer and Werck-Reichhart, 2003). In fact, the induction of CYP transcripts has previously been reported during *Fusarium* head blight resistance in wheat (Kong *et al*., 2005) and barley (Boddu *et al*., 2006), as well as during an HR in Arabidopsis resistance to *Alternaria brassicicola* (Narusaka *et al*., 2004). We found evidence for the biosynthesis of lignin and anthocyanins in only the basal defence transcripts. However, lignin biosynthetic transcripts, including cinnamyl-alcohol dehydrogenase (CAD) and dirigent-like proteins, were induced by 12 hpi in the incompatible interaction compared with 24 hpi in the compatible interaction. Subsequently, the earlier activation of lignin biosynthesis may be a result of HR-specific CYP activity, some of which were induced as early as 6 hpi. The induction of these transcripts before the hypothesized *Pst* penetration time-point (16 hpi) indicates that they may not be directly controlled by the *Yr5* gene-for-gene interaction and may reflect induced changes caused by pathogen inoculation or germination. However, it is not known whether resistance to *Pst* is pre- or post-haustorial, and induction of defence-related transcripts during pre-penetration of fungal pathogens has been observed in similar studies (Caldo *et al*., 2004; Van de Mortel *et al*., 2007). Other important transcripts observed in this study were a chromosome condensation factor and two S1/P1 nucleases that were identified as *Pst*-specific *Yr5* transcripts. Together, these observations may be indicative of the HR in *Yr5*. Recent studies of the oat–*Puccinia coronata* and Arabidopsis–*Pseudomonas syringae* interactions have identified that, adjacent to the first dying cells in the HR, neighbouring cells display apoptotic features of chromosome condensation and endonucleolytic cleavage (Greenberg and Yao, 2004). Evidence for endonucleolytic cleavage has also been found during the HR of cowpea after challenge with cowpea rust fungus (Heath, 2000). Greenberg and Yao (2004) propose that cells may die by various mechanisms in an HR; therefore, we propose that the chromosome condensation factor and nucleases found in *Yr5* may be important indicators of an effective HR mechanism during resistance to *Pst*.

In summary, the wheat transcriptional response during an incompatible and compatible interaction with *Pst* shows similar properties to other plant–pathogen interactions involving single *R* genes, including many shared transcripts (basal defence transcripts) during the initial stages of infection. We propose that the presence of *Yr5* results in a rapid and amplified resistance response involving signalling pathways and defence-related proteins known to occur during *R*-gene-mediated responses. The absence of *Yr5* does not completely remove a defence response, and in fact substantial up-regulation of many defence-related transcripts does occur in basal defence. However, the lack of *R*-gene signalling results in a weaker response that lacks 61 HR-specific induced transcripts and is unable to confer an effective HR to prevent pathogen spread. The data generated in this study provide novel insights into the cellular mechanisms of wheat defence to an economically important pathogen. The findings will be useful for understanding wheat resistance to *Pst* to enable the development of durable resistant cultivars. Future work will involve functional verification of important transcripts by reverse genetics. The data generated are publicly available on WheatPLEX and will be useful for comparative studies in both wheat and other plants.

EXPERIMENTAL PROCEDURES

Plant material and fungal isolate

Near isogenic lines (NILs) for the *Yr5* resistance gene were developed at the Plant Breeding Institute, Sydney, Australia, by backcrossing the *Yr5* donor [*Triticum aestivum* subsp. *spelta* (L.) Thell. cv. Album (TSA)], with the recurrent susceptible spring wheat genotype (*Triticum aestivum* L.) 'Avocet Susceptible' (AVS) six times (AVS*6/*Yr5*) and selecting for the appropriate resistance at each generation (Wellings *et al*., 2004). Backcrosses were advanced to the BC_7:F_4 stage (Yan *et al*., 2003), and thus only ~0.4% of the TSA genome remained in the *Yr5* NIL in the AVS background. For this study, two BC_7:F_4 NILs were selected that differed at the *Yr5* locus: *Yr5* (resistant) and y*r5* (susceptible). A highly virulent isolate (06-194) of *Puccinia striiformis* Westend. f. sp. *tritici* Eriks. identified as PST-100 (06-194) (Chen, 2005) was selected and maintained on susceptible genotypes.

Experimental design

For each of three biological replications, individual genotypes were planted in separate 25 × 42.5-cm flats using a potting mix (6 peat moss : 4 vermiculite with lime : 3 sand : 3 commercial potting mix : 2 perlite : 1.7 g/L lime : 3.3 g/L Osmocote : 2.2 g/L ammonium nitrate). Each flat consisted of ten rows of six seedlings, with rows randomly assigned one of four harvest times (6, 12, 24 and 48 hpi). Seedlings from the tenth row were used to monitor the expected disease responses to inoculation. Seedlings were grown to the second leaf stage (~10 days after planting) in a greenhouse with a diurnal temperature cycle of 10 °C (02:00 h) to 25 °C (14:00 h) and a 16-h light/8-h dark cycle. Inoculation was performed by misting the plants with sterile water and applying a 1 : 20 urediniospore/talc mixture to leaves with a sterile brush. Talc was used to aid in the spread and adhesion of spores over leaf surfaces. Control flats were treated the same way except for the absence of spores in the talc. All treatments for each biological replication were performed at 09:00 h Pacific Standard Time. To promote spore germination, all flats were

transferred to a dew chamber (100% relative humidity) operating at 10 °C in the dark for 24 h, before being placed in a growth chamber with a diurnal temperature cycle of 4 °C (02:00 h) to 20 °C (14:00 h) and a 16-h light/8-h dark cycle. Rows of plants were harvested from all flats at the assigned times for RNA extraction.

Wheat GeneChip® probe array

The GeneChip® Wheat Genome Array (Affymetrix, Santa Clara, CA) is a 3′IVT array that includes 61 127 probe sets representing 55 052 transcripts for all 21 wheat chromosomes in the genome: 59 356 probe sets represent modern hexaploid (A, B and D genomes) bread wheat (*T. aestivum*) and are derived from the public content of the *T. aestivum* UniGene Build #38 (24 April 2004); 1215 probe sets are derived from ESTs of a diploid near relative of the A genome (*T. monococcum*), a further 539 represent ESTs of the tetraploid (A and B genomes) macaroni wheat species *T. turgidum*, and five are from ESTs of a diploid near relative of the D genome known as *Aegilops tauschii*. Probe sets consisted of pairs of 11 perfect match (PM) and mismatch (MM) 25-mer oligonucleotides designed from the 3′ end of exemplar sequences, with nucleotide 13 as the MM. Each probe set was assumed to represent a transcript and array annotation information is available on the NetAffx data analysis centre (www.affymetrix.com).

Target synthesis and GeneChip hybridization

The leaves from six seedlings corresponding to each experimental condition were quickly cut, pooled and frozen in liquid nitrogen. Total RNA was extracted from 0.5 g of pooled tissue using the Trizol® Plus RNA Purification Kit (Invitrogen, Carlsbad, CA) with an on-column DNase treatment. Purified total RNA samples were quantified with a SmartSpec™ 3000 (Bio-Rad, Hercules, CA) spectrophotometer, and acceptable purity was indicated by $A_{260:280}$ ratios of 1.9–2.1 in 10 mM Tris-HCl (pH 7.5). Integrity of total RNA samples was assessed by denaturing formaldehyde gel electrophoresis, where the presence of sharp 28S and 18S ribosomal RNA bands at an intensity ratio of ~2 : 1 (28S : 18S) indicated good integrity. Labelled probes were prepared using GeneChip one-cycle target labelling and control reagents according to the manufacturer's protocol (Affymetrix). Ten micrograms of labelled cRNA was used for each hybridization. All hybridizations and data acquisition were performed at the Bioinformatics Core Facility at Washington State University according to standard Affymetrix protocols (www.bioinformatics.wsu.edu).

Data analysis

GeneChip data analysis was performed at the Bioinformatics Core Facility using both GeneChip Operating Software (GCOS) v.1.4 (Affymetrix) and GeneSifter (VizXlabs, Seattle, WA). Quality control was performed using GCOS where, first, raw intensity (DAT) files were visually inspected for scratches/smears and uniform performance of the B2 oligo around the border of each image. All cell intensity (CEL) files were then globally scaled to a target intensity of 500, and a report was generated from each result (CHP) file. Reports were examined for further quality control aspects, including average background, noise (raw Q) and percentage present to be consistent amongst all replications, and performance of the Affymetrix polyA and hybridization controls. Subsequently, hybridizations that did not satisfy these stringent quality parameters were repeated. Additionally, to confirm initial RNA quality, RNA degradation plots for each sample were generated from CEL files using the *affy* package of BioConductor (http://bioconductor.org). This function averaged probe intensities for each probe set and plotted them from 5′ to 3′ in order to identify any degradation. All individual Affymetrix CEL files were then imported into GeneSifter for normalization and analysis. Robust Multi-array Average (RMA) (Bolstad *et al.*, 2003; Irizarry *et al.*, 2003) pre-processing was performed on the entire data set, and correlation coeffcients were calculated between replications using the $\log_2$-transformed RMA-summarized data in Microsoft Excel (Microsoft, Redmond, WA). All minimum information about microarray experiments (MIAME) guidelines were observed and GeneChip data were deposited into WheatPLEX (Shen *et al.*, 2005), under accession number TA9.

Using GeneSifter, data files representing biological replications were pooled to generate mean signal values and error measurements, and projects were created to examine the data (Table 1). Within genotypes, parametric two-way ANOVA ($P < 0.001$) tests, assuming equal variance, with FDR (Benjamini and Hochberg, 1995) multiple testing correction at 5% were performed on $\log_2$-transformed RMA-summarized data to detect differentially expressed probe sets by treatment (*Pst* inoculated or mock-inoculated), time and treatment × time interaction. Between genotypes, the same tests were performed to detect differentially expressed probe sets by genotype (*Yr5* or *yr5*), time and genotype × time interaction. Additionally, for all tests, differentially expressed probe sets must have possessed a fold change > 2.0 to be considered. For all data comparisons, differentially expressed probe sets significant for the time effect only were excluded from further analysis. The unigenes for each differentially expressed probe set were annotated using HarvEST (Affymetrix Wheat1 Chip version 1.52), which provided the current best BLASTX hit from the non-redundant (nr) database of NCBI, as well as the best BLASTX hits from rice and Arabidopsis TIGR databases. A database hit of < 1e-10 was considered as significant, or otherwise the unigene was annotated as unknown. For GO, the rice locus matching each probe set in the HarvEST output was queried using the TIGR rice genome annotation (Yuan *et al.*, 2003), which provided GO terms including biological function.

Quantitative RT-PCR

Twenty-three target probe sets were selected for confirmation of GeneChip expression ratios by quantitative RT-PCR. The sequence of the unigene corresponding to each probe set was identified in HarvEST. Forward and reverse primers for quantitative detection were designed using PrimerQuest (Integrated DNA Technologies, www.idtdna.com) with the 'real-time' parameter set. For each isoline, treatment and time-point, 1.0 μg total RNA from one biological replication used for GeneChip hybridization was converted to cDNA template using random hexamers and iScript reverse transcriptase (Bio-Rad) according to the manufacturer's instructions. The resulting cDNA products were diluted to 200 μL in sterile water. A Histone H3.2 unigene from Affymetrix probe set Ta.822.1.S1_x_at, whose expression remained consistent amongst all experimental conditions, was selected as the normalization gene for relative quantification of the target probe sets. Before proceeding with quantitative PCR, validation of each primer pair was performed by: (1) selecting one cDNA sample and creating a dilution series over four orders of magnitude, (2) performing separate real-time PCR on the dilution series with the normalization primer pair and all target primer pairs, and (3) calculating the efficiency of amplification across the dilution series with the iQ5 Optical System software v.2.0 (Bio-Rad). To pass validation, each primer pair must have demonstrated 85–100% amplification efficiency. Triplicate quantitative RT-PCRs were then performed on experimental samples using iQ Sybr Green Supermix (Bio-Rad) with primers (400 nM each) and 5 μL of cDNA. Control reactions containing untranscribed RNA confirmed that no interfering genomic DNA products were present. PCR was performed on a Bio-Rad iQ5 Real-Time PCR Detection System instrument (Bio-Rad) with the following cycling programme: 95 °C for 3 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. All products were subjected to melting curve analysis and verified by gel electrophoresis. Relative fold changes were calculated by the comparative C_T method ($\Delta\Delta C_T$ method).

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

Table S1 List of the significant incomplete isogenicity transcripts at one or more time-points after *Puccinia striiformis* f. sp. *tritici*- and mock-inoculation following two-way ANOVA ($P < 0.001$, genotype effect only), FC > 2.0 cutoff, and FDR correction. Values represent mean $\log_2$ FC of *Yr5* in reference to *yr5* such that positive values indicate probe sets expressed at a higher level in *Yr5* and negative values are those at a higher level in *yr5* samples. GO refers to gene ontology, and putative function represents the best database hit from HarvEST.

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